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Targeted Disruption of the Gamma-Synuclein Activated  
Migratory and Survival Signaling Pathways

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<b>13. ABSTRACT (Maximum 200 Words)</b> <p>Synucleins are a family of highly conserved small proteins that are normally expressed predominantly in neurons. Very little is known about the physiological functions of the synucleins. We have reported that g-synuclein (also known as BCSG1) is dramatically up-regulated in the vast majority (&gt;70%) of late-stage breast and ovarian cancers (Bruening, et al., 2000). When over-expressed, g-synuclein significantly stimulates cell proliferation and metastasis in some breast cancer cell lines. We have shown that DNA hypomethylation is a common mechanism underlying the abnormal expression of this gene in tumor cells (Gupta et al., 2003) and hypothesize that g-synuclein may be a proto-oncogene and that aberrant expression of this protein may contribute to the development and progression of ovarian cancer. We also found that g-synuclein can promote cancer cell survival and inhibit stress- and chemotherapy drug-induced apoptosis by modulating MAPKs. Specifically, over-expression of g-synuclein lead to constitutive activation of ERK1/2, and down-regulation of JNK1 in response to a host of environmental stress signals, including UV, heat shock, sodium arsenate, nitric oxide and chemotherapeutic drugs (Pan, Z-Z, et al., 2002). Because of its high frequency of expression in late-stage ovarian cancers, we hypothesized that g-synuclein may be a promising target for cancer therapy.</p>				
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## INTRODUCTION:

The synucleins ( $\alpha$ ,  $\beta$ ,  $\gamma$ , synoretin) are a family of highly conserved small proteins that are normally expressed predominantly in neurons. Little is known about the normal functions of synucleins in physiological conditions. Of the synucleins,  $\alpha$ -synuclein is the best characterized because of its potential significance in neurodegenerative diseases including Parkinson's Disease. Recently, we and others have found that  $\gamma$ -synuclein is dramatically up-regulated in the vast majority of late-stage breast (70%) and ovarian (>85%) cancers and that  $\gamma$ -synuclein over-expression can enhance tumorigenicity (Bruening et al., 2000; Ji et al., 1997; Liu et al., 2000). We also observed that expression of  $\gamma$ -synuclein induces a phenotype similar to that induced by activation of RhoA/Rac/CDC42, altering the appearance of focal adhesions and stress fibers, and enhancing motility and invasion in ovarian cancer cells (Pan et al, submitted 2004). Recent studies have also shown that when  $\gamma$ -synuclein is overexpressed in a breast tumor derived cell line, the cells experience a dramatic augmentation in their capacity to metastasize *in vivo* (Jia et al., 1999). Based on these known data, we hypothesize that  $\gamma$ -synuclein may be a proto-oncogene and that aberrant expression of this protein may contribute to the progression of ovarian cancer and that this tumor-associated protein may be promising targets for drug discovery. We further hypothesize that  $\gamma$ -synuclein may be promoting this phenotype in part by activating the RhoA signal transduction pathway and have shown that RAC as well as the mitogen-activated kinase, ERK1/2, are constitutively activated in cells that overexpress  $\gamma$ -synuclein, but not alpha or beta. Furthermore, we have found that the activation of JNK by stress signals is significantly down-regulated by  $\gamma$ -synuclein, suggesting a role in tumor cell survival and inhibition of apoptosis. If expression of  $\gamma$ -synuclein in tumor cells induces an invasive phenotype and promotes tumor cell survival, then understanding how  $\gamma$ -synuclein functions may lead to therapies for metastatic disease. Thus, drugs that block the action of  $\gamma$ -synuclein may inhibit the spread of ovarian cancer, as well as other neoplasms. We propose to further define the function of  $\gamma$ -synuclein through studying the signaling pathways that it affects. Disrupting the interactions between  $\gamma$ -synuclein and its interactors may provide a means to limit tumor cell metastasis, while inducing limited or no toxicity among other cells in human adults.

## BODY

Progress report year 2.

Task 1 (Months 1-24). Determine how  $\gamma$ -synuclein modulates JNK and ERK signaling transduction and its effects on cell survival, apoptosis and tumor progression.

As indicated in the previous annual progress report (4-04), we have addressed the objectives of this task.

Task 2 (Months 3-36). Identify specific mutations, and small molecule agents, that specifically disrupt interactions between  $\gamma$ -synuclein and ERK1/2 and between  $\gamma$ -synuclein and JNK, and evaluate them in cell culture models, with a goal of developing them as targeted therapeutics.

We have continued to make progress in regards to this task. We have identified several small peptides that bind to  $\gamma$ -synuclein, and are currently characterizing their actions *in vitro*. We have also identified additional proteins that interact with  $\gamma$ -synuclein that can be used to screen the small peptides for functional activity. The state of these studies is detailed below.

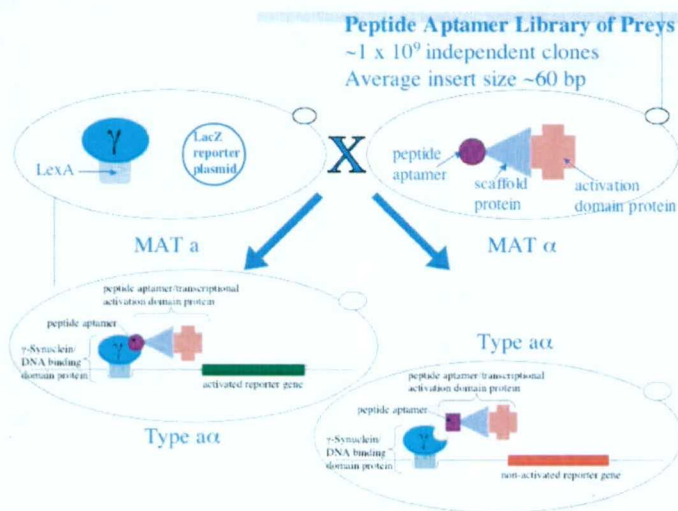


**Task 1-Progress Report**—"Determine how  $\gamma$ -synuclein modulates JNK and ERK signaling transduction and its effects on cell survival, apoptosis and tumor progression". See progress report-year 1.

**Task 2-Progress Report**—"Identify specific mutations, and small molecule agents, that specifically disrupt interactions between  $\gamma$ -synuclein and ERK1/2 and between  $\gamma$ -synuclein and JNK, and evaluate them in cell culture models, with a goal of developing them as targeted therapeutics".

There is an urgent need for effective therapies for women with advanced stage breast and ovarian cancers. One method to develop new therapies is to identify proteins that are over expressed in these cancers and to then derive inhibitors to their function(s). This provides a two pronged approach to drug development in that it has the potential to both (A) make the cancer cells more susceptible to traditional cancer therapies [radiation and chemotherapy] and (B) provide a free standing new treatment regimen using protein or nucleic acid derived drugs which have the promise of being both more effective, and having fewer side effects than traditional cancer therapies. Recently,  $\gamma$ -synuclein has been shown to be over expressed in the majority of late stage breast and ovarian cancers, but not in early stage cancers or in normal breast and ovarian tissues (1, 2).  $\gamma$ -Synuclein has also been shown to enhance the drug resistance and metastatic capability of breast and ovarian cancer cells (3, 4).  $\gamma$ -Synuclein may increase drug resistance of cancer cells by interfering with MAP kinase pathways which in turn, increases cellular resistance to chemotherapeutic drugs that function by activating the JNK mediated apoptosis pathway (3).  $\gamma$ -Synuclein may increase cancer cell metastasis by up-regulating the production of matrix

metalloproteinases, which are involved in rebuilding of the extracellular matrix (5). We have utilized yeast two-hybrid methodology to screen for protein interactors to  $\gamma$ -synuclein in an effort to further elucidate its role in cell signaling pathways, and have also used this methodology to screen for small peptide inhibitors to  $\gamma$ -synuclein with the goal of developing these inhibitors into novel therapies for the treatment of advanced stage breast and ovarian cancers. In addition, we have established a working collaboration with the laboratory of Dr. Russell Finley (Wayne State University School of Medicine, Detroit, MI) to screen for additional interactors to  $\gamma$ -synuclein using a *Drosophila melanogaster*-derived cDNA library of proteins in a yeast two-hybrid based assay. The human homolog(s) of any verifiable  $\gamma$ -synuclein-interacting *Drosophila* proteins will be further investigated.




**Figure 1.** Schematic representation of the peptide screening approach. Yeast containing the synuclein bait are mated with yeast possessing one of the  $\sim 1 \times 10^6$  unique peptide prey expression constructs. Peptides that are able to interact with synuclein are identified and the sequence determined.

Putative  $\gamma$ -synuclein interacting peptide aptamers and control vectors were validated for interaction with  $\gamma$ -synuclein using the yeast two-hybrid system. A total of 324  $\gamma$ -synuclein bait strain/peptide aptamer cDNA library minus leucine positive diploid yeast clones were discovered by mating a  $\gamma$ -synuclein/DNA binding domain LexA fusion vector transformed strain of haploid yeast with a peptide aptamer cDNA library transformed haploid strain of yeast of the opposite mating type. Of these 324 clones, 53 (16%) demonstrated reproducible interactions with  $\gamma$ -synuclein (**Figure 1**).



Forty-nine of these clones were successfully sequenced. Of these 49 clones, 47 had one or more premature translational termination codons in close proximity to the N-terminal region of the thioredoxin active site coding sequence, which raised the question as to whether or not thioredoxin or a portion of thioredoxin may be a potential  $\gamma$ -synuclein interactor (**Figure 2**). The peptide aptamer is expressed as a

#### $\gamma$ -Synuclein/Peptide Aptamer Interactor Representative Sequencing Results



* 69-1	NAEWCDF	SLGS*RAFFLGHGEENKQL	GPCKMIAP
* 71-5	NAEWCDF	SLGS*RAFFLGHGEENKQL	GPCKMIAP
* 117-3	NAEWCDF	RE*SIAMHSLGLECVYTH	GPCKMIAP
* 132-8	NAEWCDF	RE*SIAMHSLGLECVYTH	GPCKMIAP
* 265-4	NAEWCDF	RE*SIAMHSLGLECVYTH	GPCKMIAP
* 268-3	NAEWCDF	RE*SIAMHSLGLECVYTH	GPCKMIAP
* 274-7	NAEWCDF	RE*SIAMHSLGLECVYTH	GPCKMIAP
* 275-4	NAEWCDF	RE*SIAMHSLGLECVYTH	GPCKMIAP
* 282-7	NAEWCDF	VL*APAHNGTQSSSTAYL	GPCKMIAP
* 283-8	NAEWCDF	VL*APAHNGTQSSSTAYL	GPCKMIAP
* 289-7	NAEWCDF	VL*APAHNGTQSSSTAYL	GPCKMIAP
* 5-8	NAEWCDF	V*ATNCIAQHSKVFLLAC	GPCKMIAP
* 59-2	NAEWCDF	VQ*FCSLGHGHEHT*FQLGE	GPCKMIAP
* 59-4	NAEWCDF	WV*VAKAGGSESFR*FPRV	GPCKMIAP
* 122-4	NAEWCDF	L*WVWGGIAYLTDGROFF	GPCKMIAP

**Figure 2.** Examples of the sequence of the peptide aptamers found to interact with  $\gamma$ -synuclein through yeast two-hybrid screening approaches. Note that redundant clones were independently isolated during these screens, suggesting that they are likely to be genuine synuclein interactors. \*=stop codon.

fusion protein that contains a nuclear localization sequence, a transcriptional activation domain, a haemagglutinin (HA) epitope tag, an N-terminal thioredoxin A sequence (amino acid 1-35), and a C-terminal thioredoxin A sequence (amino acid 36-110) (Accession #M54881 M38747). The segment of thioredoxin that immediately precedes expression of the aptamer, i.e. the region that actually displays the aptamer, is the active site portion of the thioredoxin protein (6). This expression can be represented as follows, where the underlined region represents the thioredoxin active site sequence.

WAEWCGP – aptamer – GPCKMIAP

This active site sequence is well conserved between *E. coli* and humans (6). The complete protein sequence of this region is WCGPC, which covers amino acid residues 32-36 of the thioredoxin protein [Accession #M54881 M38747, (6)].

Interestingly, while most of the putative  $\gamma$ -synuclein interacting aptamer clones contained all of the above-mentioned sequence elements, the majority (~96%) lacked a complete aptamer sequence. In addition, some truncated aptamer clones were identified multiple times, further supporting the possibility for a verifiable interaction with  $\gamma$ -synuclein. In these clones, the epitope for interaction with  $\gamma$ -synuclein could have been a combination between the N-terminal thioredoxin sequence and the truncated aptamer, or possibly the N-terminal thioredoxin sequence itself. Interaction with  $\gamma$ -synuclein could also have been due to an association between  $\gamma$ -synuclein and the entire N-terminal thioredoxin, aptamer, C-terminal thioredoxin sequence combination, or it could have been simply due to an association between  $\gamma$ -synuclein and the HA tag part of the fusion protein.

In order to sort out all of these potential possibilities, we have generated different aptamer constructs to delineate the exact nature of the interaction between  $\gamma$ -synuclein and selected aptamer clones using yeast two-hybrid methodology (**Figure 3**). Constructs ranged in sequence coverage from the nuclear localization sequence to the C-terminal thioredoxin region (**Figure 4A**), from the nuclear localization sequence to the N-terminal thioredoxin region (**Figure 4B**), and from the nuclear localization sequence to the HA tag region (**Figure 4C**). An N-C terminal thioredoxin construct containing no aptamer sequence was also generated (**Figures 5-6**) for use as a control for interaction with thioredoxin. In this way, all possible interactions between  $\gamma$ -synuclein and isolated aptamers are being investigated. The peptide aptamer derived constructs that most strongly interact with  $\gamma$ -synuclein will be further studied.

We chose the pYES2.1 TOPO cloning vector (Invitrogen) for validation of peptide aptamer derived constructs for interaction with  $\gamma$ -synuclein. This vector contains a *GALI* promoter followed by a cloning site and a *CYCI* transcriptional termination region, but does not contain a nuclear localization sequence, a transcriptional activation domain, or a HA-epitope tag. For consistency in experimental design, each of the aptamer derived transcriptional activation domain fusion proteins being tested needed to be expressed from the same plasmid vector backbone. The cDNA library vector that originally housed each peptide aptamer clone, i.e. the “pJM-1” vector, (**Figure 3**) was unavailable in an aptamerless form. Extensive



restriction analysis has also indicated that there are no available sites for complete removal of aptamer sequences from individual clones. Therefore, it was not possible to use the pJM-1 vector for sequence-specific validation of putative  $\gamma$ -synuclein binding clones. However, it was possible to transfer designated sequences into a galactose-inducible, transcriptional termination region-containing vector that has no native transcriptional activation domain, i.e. the pYES2.1 TOPO vector. In this way, only desired aptamer derived sequences are being tested for interaction with  $\gamma$ -synuclein.

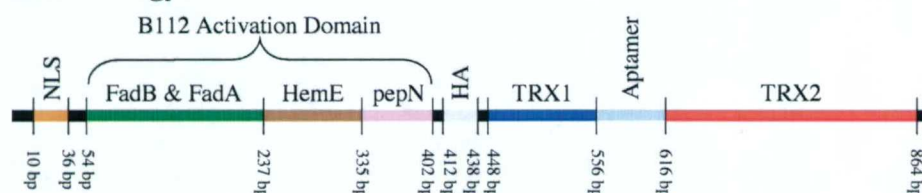
### **$\gamma$ -Synuclein interaction validation using putative $\gamma$ -synuclein interacting peptide aptamer derived constructs and control vectors with the yeast two-hybrid system.**

#### **A. Determination of aptamer library pJM-1 vector sequence.**

The peptide aptamer cDNA library is housed in a vector construct termed "pJM-1." The nature of the transcriptional activation domain and the complete sequences of the thioredoxin coding regions of this vector was previously unknown by our laboratory. There was some confusion regarding the exact makeup of these regions of the vector. To resolve this issue, several pJM-1 vectors were completely sequenced to identify components immediately preceding and following the *GAL1* promoter region. Blast analysis was performed on segments of DNA from this region. In this way, the nuclear localization sequence and each component of the transcriptional activation domain was identified. The same pJM-1 vectors were also sequenced to identify components preceding the transcriptional termination region. Blast analysis was also performed on segments of DNA from this region. Using this methodology, the complete C-terminal thioredoxin sequence, individual peptide aptamer sequences, the complete N-terminal thioredoxin sequence, and the haemagglutinin tag sequence were each identified (**Figure 3**).

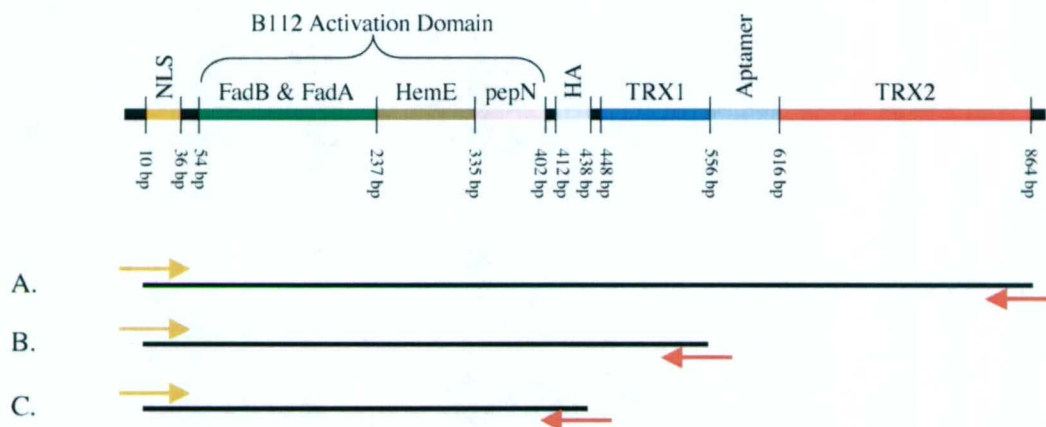
The pJM-1 vector was found to contain a transcriptional activation domain that consisted of three distinct components – *E. coli* Fad B and Fad A genes [abbreviated "FadB & FadA"]; *E. coli* hemE gene for uroporphyrinogen III decarboxylase [abbreviated "HemE"]; and *E. coli* pepN gene encoding aminopeptidase N [abbreviated "pepN"]. Combined together, these components are collectively called the B112 transcriptional activation domain (7). From this information, the following schematic of the pJM-1 vector was generated:

#### **B. PCR-based generation of vectors for validation of interaction with $\gamma$ -synuclein using yeast two-hybrid methodology.**



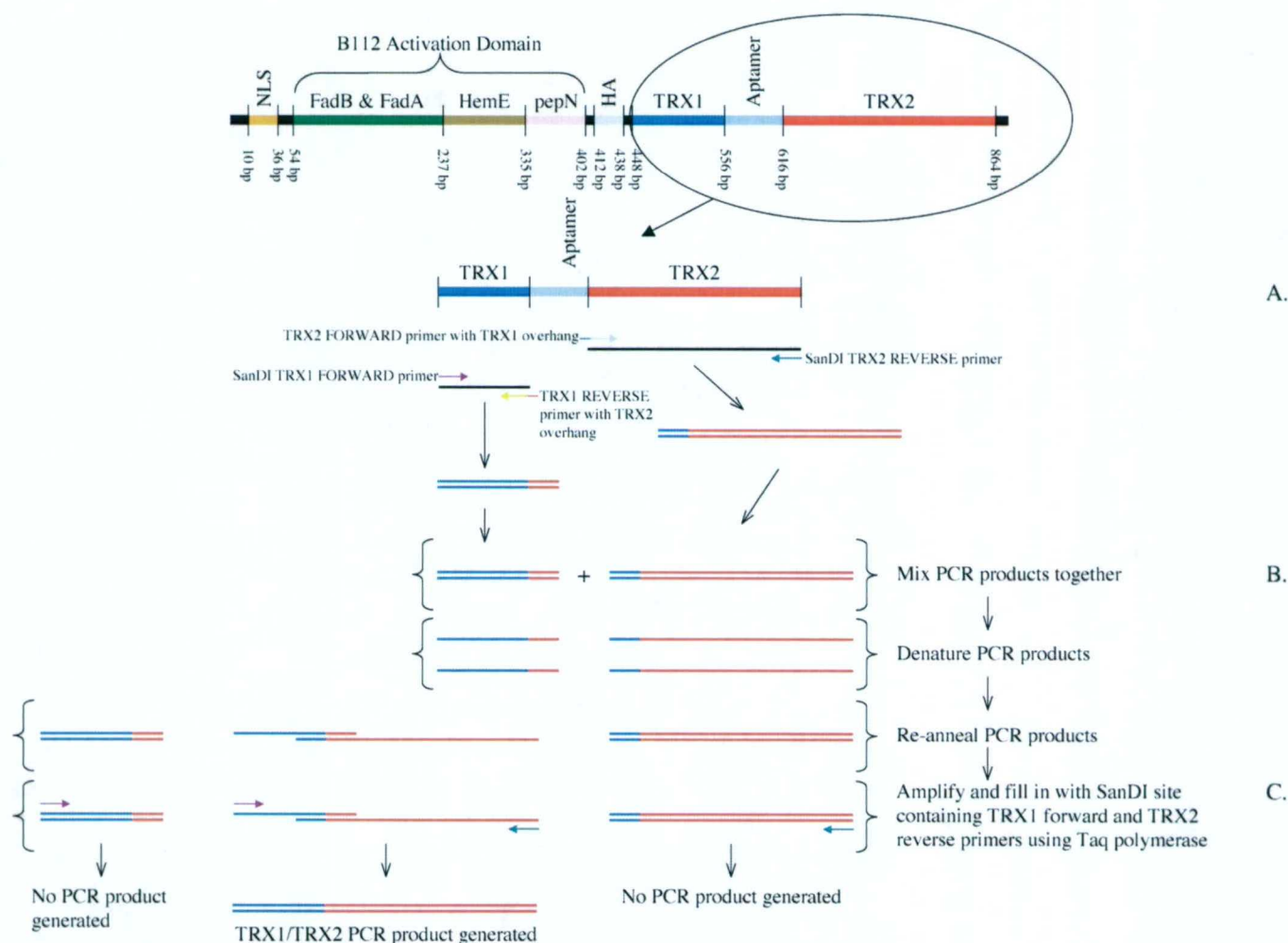
**Figure 3. Diagrammatic representation of the peptide aptamer library pJM-1 vector.** The vector contains a *GAL1* promoter followed by a nuclear localization sequence [NLS], a B112 transcriptional activation domain consisting of *E. coli* Fad B and Fad A genes [FadB & FadA]; *E. coli* hemE gene for uroporphyrinogen III decarboxylase [HemE]; and *E. coli* pepN gene encoding aminopeptidase N [pepN]. The vector also consists of a haemagglutinin tag [HA], an *E. coli* derived N-terminal thioredoxin A sequence [TRX1], a peptide aptamer sequence [Aptamer], and an *E. coli* derived C-terminal thioredoxin A sequence [TRX2] followed by a transcriptional termination region.

**C. PCR-based generation of complete, uninterrupted thioredoxin DNA fragment for validation of interaction with  $\gamma$ -synuclein.**



**Figure 4. Generation of clones for determination of pJM-1 vector derived specificity of interaction with  $\gamma$ -synuclein using yeast two-hybrid methodology.** All of the two-hybrid reactions must take place in the nucleus of the yeast, so each construct contained a nuclear localization sequence and ranged in coverage from the nuclear localization sequence to the C-terminal thioredoxin sequence. Because 47 of the 49 putative  $\gamma$ -synuclein interacting peptide aptamer clones contained one or more early translational termination codons, the specificity of each clone's interaction with  $\gamma$ -synuclein is being investigated. In all constructs (A, B, and C), the amplified sequence began with a 5' pJM-1 vector-native yeast translational initiation sequence followed by a nuclear localization sequence and spanned to the C-terminal thioredoxin region (A), the N-terminal thioredoxin region (B), or the HA tag region (C). PCR products were purified from 2% agarose gel and transferred directly into the pYES2.1 TOPO vector. Restriction analysis with XbaI enzyme was used to confer correct orientation of insert sequences. A total of 10 type A constructs were produced, while 1 type B construct and 1 type C construct have also been generated.



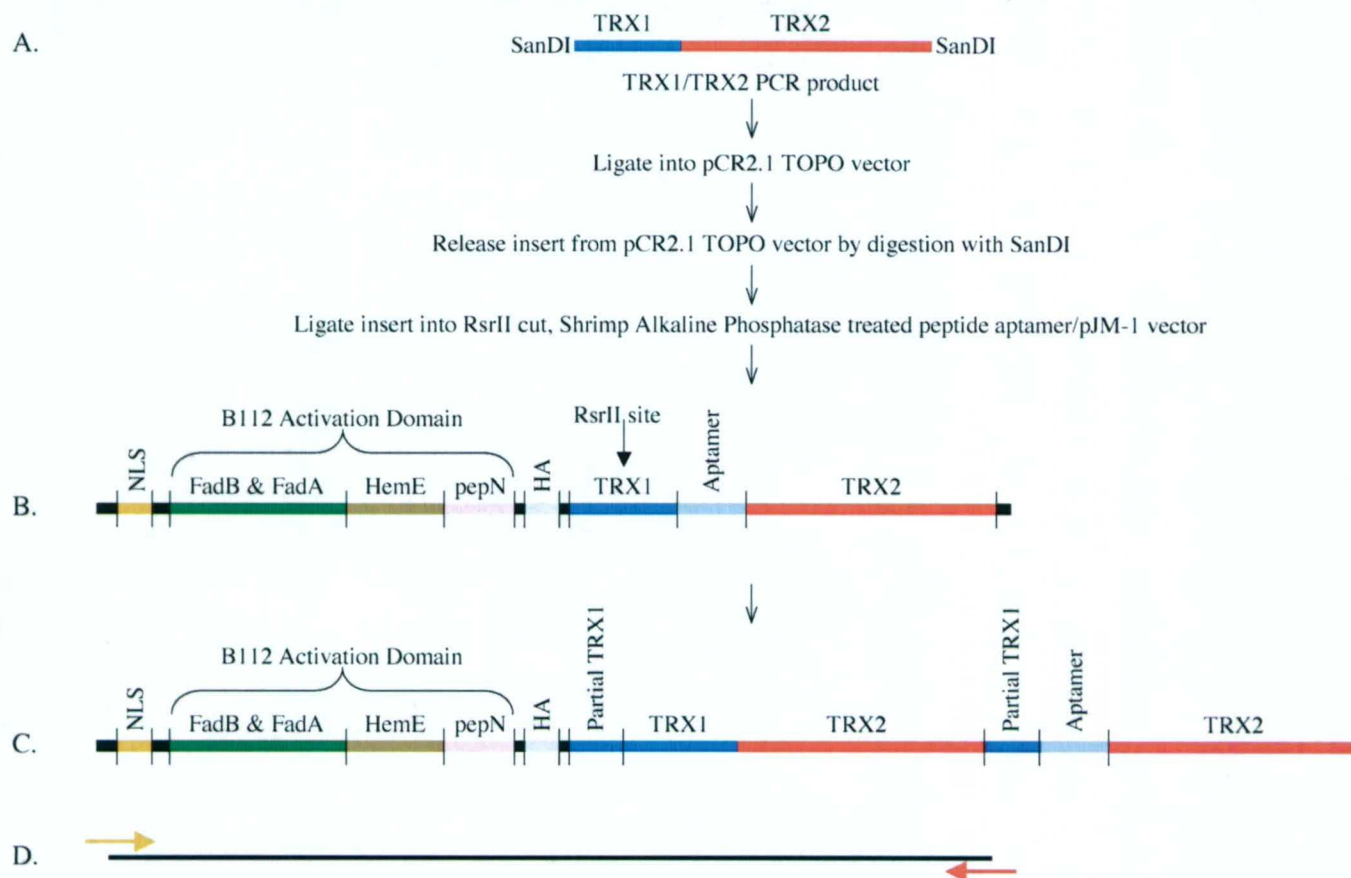


**Figure 5. Generation of non-peptide aptamer containing thioredoxin DNA sequence for transfer into yeast expression vector.** Peptide aptamer coding sequence must be removed from the N- and C-terminal thioredoxin sequences in order to generate the DNA fragment necessary to create an *E. coli* sequence-derived thioredoxin control vector. Due to the unavailability of an aptamerless pJM-1 vector, this procedure was used to generate a non-aptamer containing construct. Generation of the complete thioredoxin sequence was a three step PCR-based process. In step (A), the N-terminal thioredoxin sequence [TRX1] was amplified using a forward primer containing a SanDI restriction site sequence and a reverse primer containing a short C-terminal thioredoxin sequence [TRX2] overhang. The resulting N-terminal thioredoxin [TRX1] PCR product contained a 5' SanDI restriction site and a 3' C-terminal thioredoxin [TRX2] overhang. The C-terminal thioredoxin sequence [TRX2] was independently amplified using a forward primer with an N-terminal thioredoxin [TRX1] overhang and a reverse primer containing a SanDI restriction site sequence. The resulting C-terminal thioredoxin [TRX2] PCR product contained a 5' N-terminal [TRX1] overhang and a 3' SanDI restriction site. In steps (B and C), the two PCR products resulting from step (A) were mixed together, denatured, re-annealed (step B) and used as templates for amplification with the SanDI restriction site containing N-terminal [TRX1] forward primer and the SanDI restriction site containing C-terminal [TRX2] reverse primer (step C). It is important to note that in step (B), three different template products resulted – two of the three products were re-annealed PCR products identical to those generated in step (A). The third template was a heteroduplex product that formed between the N-terminal C-terminal overhang PCR product and the C-terminal N-terminal overhang PCR product. This was the only template that could be successfully amplified using



the SanDI restriction site containing N-terminal [TRX1] forward/SanDI restriction site containing C-terminal [TRX2] reverse primer pair. Taq polymerase both filled in and amplified this template. In this way a complete N-C terminal thioredoxin sequence was generated that contained no peptide aptamer insert. The product contained 5' and 3' SanDI sites to allow insertion into an RsrII cut pJM-1 library vector (as described in Figure 4). RsrII sequences could not be included in the TRX1 forward primer and the TRX2 reverse primer because the final PCR product (step C) contained an internal RsrII restriction site. Cleavage with RsrII enzyme would fragment this product. To prevent this potential problem, SanDI restriction sequence was used in place of RsrII sequence in the above-mentioned forward and reverse primers. SanDI restriction sequence is compatible for cloning into an RsrII restriction site, and the final PCR product (step C) did not contain any SanDI sequences.

#### D. Generation of thioredoxin/pJM-1 PCR template vector and PCR-based generation of thioredoxin control vector for investigation/validation of interaction with $\gamma$ -synuclein.



**Figure 6. Transfer of complete thioredoxin N-C-terminal DNA fragment into linearized pJM-1 vector for creation of PCR template vector and generation of *E. coli* sequence derived thioredoxin clone for investigation/validation of interaction with  $\gamma$ -synuclein.** The PCR product generated in Figure 3C was transferred into RsrII-linearized, shrimp alkaline phosphatase treated aptamer-containing pJM-1 vector. The product was first cloned into the pCR2.1 TOPO vector (Invitrogen), sequence verified (DNA Sequencing Facility, Fox Chase Cancer Center, Philadelphia, PA), released from the pCR TOPO vector by digestion with SanDI, and ligated into RsrII cut, shrimp alkaline phosphatase treated peptide aptamer/pJM-1 vector (A-B). The resulting construct had a complete *E. coli* derived thioredoxin sequence downstream of the HA tag (C). This vector also had a complete peptide aptamer along with associated C-terminal thioredoxin and transcription termination regions. These sequences were irrelevant, however, because they were not utilized in generation of the final thioredoxin control vector. This vector was used as PCR template for creation of thioredoxin control vector (D). The amplified sequence began

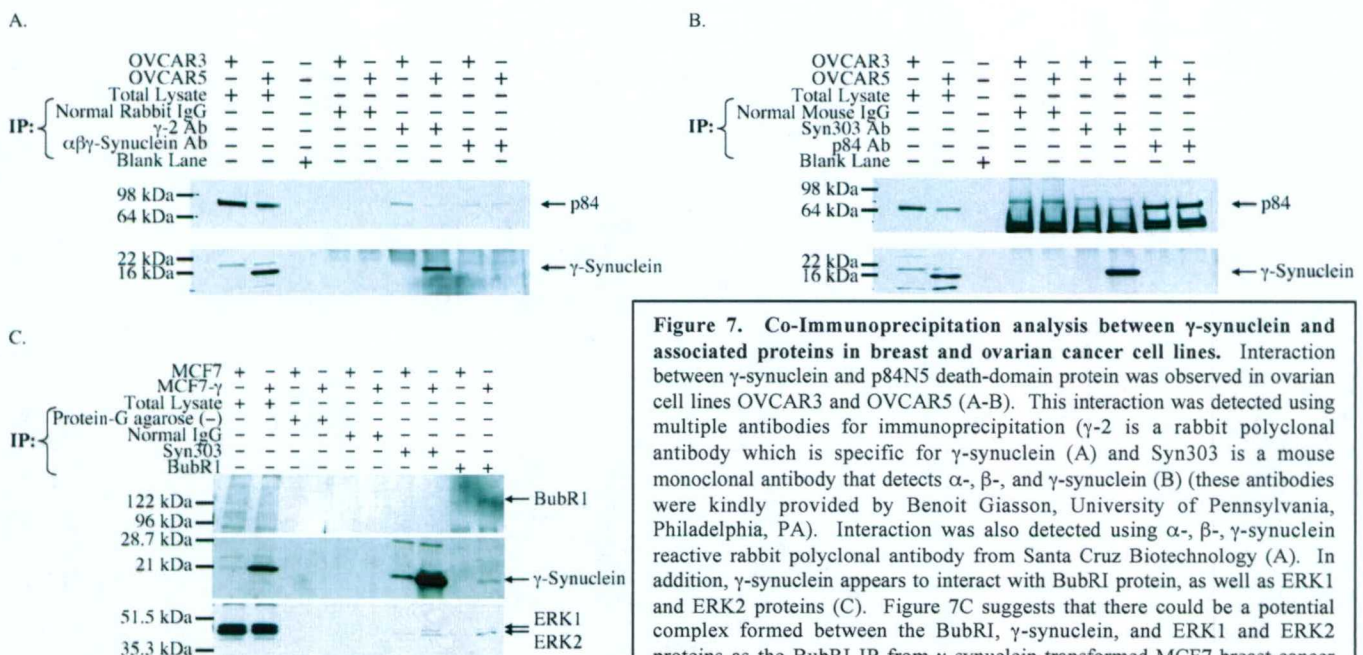


with a 5' pJM-1 vector-native yeast translational initiation sequence followed by a nuclear localization sequence and extended to the C-terminal thioredoxin region (D). The PCR product was purified from 2% agarose gel and transferred directly into the pYES2.1 TOPO vector. Restriction analysis with XbaI enzyme verified that the insert was in the correct orientation. A total of 1 type D thioredoxin control construct has been generated.

#### E. Optimization of growth and protein production using *Saccharomyces cerevisiae* yeast strain SKY473 (MATa *ura3 trp1 his3 2LexAop-LEU2 3cIop-LYS2*).

The growth and protein-production potential of SKY473 yeast has been optimized. This strain of yeast is being used to express the 13 aptamer constructs described in Sections A-D. Optimization was necessary to ensure that the yeast produced as much protein as possible. A time course was undertaken in which yeast concentration and protein production were measured at corresponding time points. Untransformed virgin yeast was grown in induction medium supplemented with complete amino acids. At the same time, yeast transformed with empty pYES2 vector (Invitrogen) and yeast transformed with *LACZ* insert containing pYES2.1 TOPO vector (Invitrogen) were cultured in induction medium minus uracil. Optimal protein production for untransformed virgin yeast was observed at 8 hours (Figure 5A-B). The highest protein production for yeast transformed with empty pYES2 vector or *LACZ* containing pYES2.1 TOPO vector was observed at 12 hours. Growth curves were nearly identical for both pYES vector transformed yeast cultures, while the virgin yeast culture exhibited an elevated growth potential. For all experimental procedures, SKY473 yeast will be grown for a minimum of 8-12 hours to ensure adequate expression from pYES2.1 TOPO vector constructs. Expression of *LACZ* from the *LACZ* pYES2.1 TOPO vector was confirmed by western blot analysis (data not shown).

#### F. Verification of $\gamma$ -synuclein protein interaction using co-immunoprecipitation analysis with human breast and ovarian cancer cell lines.



**Figure 7. Co-immunoprecipitation analysis between  $\gamma$ -synuclein and associated proteins in breast and ovarian cancer cell lines.** Interaction between  $\gamma$ -synuclein and p84N5 death-domain protein was observed in ovarian cell lines OVCAR3 and OVCAR5 (A-B). This interaction was detected using multiple antibodies for immunoprecipitation ( $\gamma$ -2 is a rabbit polyclonal antibody which is specific for  $\gamma$ -synuclein (A) and Syn303 is a mouse monoclonal antibody that detects  $\alpha$ -,  $\beta$ -, and  $\gamma$ -synuclein (B) (these antibodies were kindly provided by Benoit Giasson, University of Pennsylvania, Philadelphia, PA). Interaction was also detected using  $\alpha$ -,  $\beta$ -,  $\gamma$ -synuclein reactive rabbit polyclonal antibody from Santa Cruz Biotechnology (A). In addition,  $\gamma$ -synuclein appears to interact with BubR1 protein, as well as ERK1 and ERK2 proteins (C). Figure 7C suggests that there could be a potential complex formed between the BubR1,  $\gamma$ -synuclein, and ERK1 and ERK2 proteins as the BubR1 IP from  $\gamma$ -synuclein-transformed MCF7 breast cancer cells pulled down  $\gamma$ -synuclein, ERK1 and ERK2 proteins.

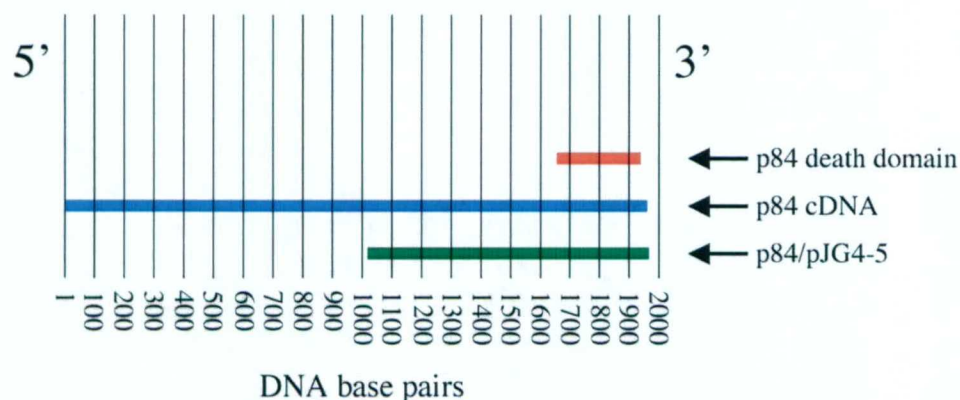
Along with preparation of the 13 yeast vectors and optimization of yeast growth and protein production potentials (described in Sections A-E), human breast and ovarian cancer cell lines have been used to test the strength of a number of proposed interactions with  $\gamma$ -synuclein. Through these studies, we



have found that  $\gamma$ -synuclein has demonstrated some interaction with p84N5 death domain protein (**Figure 7A-B**), BubRI protein, and ERK1 and ERK2 proteins (**Figure 7C**). One or more of these proteins will be utilized for testing the degree of ability that the peptide aptamer derived constructs have for disrupting interactions between  $\gamma$ -synuclein and partner proteins. We have recently reported that p84N5 also known as TREX84 is a prognostic factor in breast cancer and is essential for mRNA transport from the nucleus to the cytoplasm (Guo, et al, Cancer Research, in press, 2005). The potential role that  $\gamma$ -synuclein has regulating p84N5 function could potentially lead to a breakthrough in the understanding of the many roles that  $\gamma$ -synuclein has in increasing the invasiveness and drug resistance of breast and ovarian cancer cells and clearly warrants further investigation.

Further evidence to support the interaction between  $\gamma$ -synuclein and p84N5 came as a result of a study we performed in which a fetal brain derived cDNA library (kindly provided by Erica Golemis, Fox Chase Cancer Center, Philadelphia, PA) was screened for potential interactors to  $\gamma$ -synuclein using yeast two-hybrid methodology. In this screen, p84N5 protein was identified. The fetal brain library contained partial C-terminal protein sequences. DNA from the interacting yeast patch was isolated and the fetal brain library vector was purified and sequenced (DNA Sequencing Facility, Fox Chase Cancer Center, Philadelphia, PA). This partial sequence was aligned with p84N5 cDNA sequence [Accession #XM008756] using MacVector DNA analysis software (Accelrys). The following table was created using data generated from this analysis.

### p84 DNA alignment map

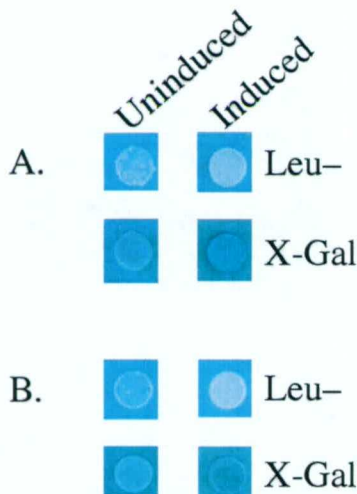


**Figure 8. DNA alignments between p84 cDNA sequence and p84/pJG4-5 library vector sequence isolated from a yeast two-hybrid screen performed by mating  $\gamma$ -synuclein transformed *S. cerevisiae* yeast EGY48 (MAT $\alpha$  *ura3 trp1 his3* 6LexAop-*LEU2*) with fetal brain cDNA library transformed *S. cerevisiae* yeast SKY473 (MAT $\alpha$  *ura3 trp1 his3* 2LexAop-*LEU2* 3clop-*LYS2*). The yeast screen isolated p84 that aligns with the C-terminal region of the p84 protein and contains the death domain region. About 50% of the p84 total sequence is present in the yeast-isolated vector (shown in green).**

Investigation of the aptamer/ $\gamma$ -synuclein/partner protein interactions in cancer cell lines will be carried out after the best candidate aptamer constructs are isolated through extensive yeast two-hybrid analyses (currently in progress).  $\gamma$ -Synuclein-interacting *Drosophila* homologs potentially identified from our collaboration with Dr. Russell Finley (Wayne State University School of Medicine, Detroit, MI) will also be considered as potential candidates for disruption with aptamer derived peptides. Preliminary data has been obtained from this collaboration (**Figure 9**).  $\gamma$ -Synuclein bait plasmid was constructed by inserting a full-length cDNA  $\gamma$ -synuclein coding region in-frame with the ADH1p-LexA (DNA binding domain) expression cassette in the pEG202 vector. To ensure that the bait would be expressed as a fusion



protein, the first ATG sequence in the  $\gamma$ -synuclein coding region was removed prior to its transfer into pEG202. Expression from the pEG202 vector was constitutively driven by the ADH1 promoter. The protein was expressed as a LexA- $\gamma$ -synuclein fusion. The prey vector library was composed of approximately 13,000 of the known open reading frame *Drosophila* sequences. The open reading frame sequences were originally amplified by the polymerase chain reaction (PCR) and cloned into galactose-inducible activation domain prey vectors. Proteins were expressed as transcriptional activation domain fusions. Resulting constructs were used to transform *S. cerevisiae* yeast. Aliquots of each were mixed to create the open reading frame prey library. Theoretically, almost every predicted open reading frame *Drosophila* sequence should be represented in this prey vector library.



**Figure 9. Preliminary data from mating  $\gamma$ -synuclein DNA binding domain bait vector transformed *S. cerevisiae* yeast with open reading frame transcriptional activation domain prey vector transformed *S. cerevisiae* yeast.** Two potential interactors (A and B) have been identified. The left column indicates yeast grown under uninduced conditions, where expression from the transcriptional activation domain prey vector is not occurring. The right column indicates yeast grown under induced conditions, where expression from the prey vectors is taking place. Leu- indicates a growth assay in which the amino acid leucine is omitted from the plates that the yeast are grown on. Interaction between  $\gamma$ -synuclein and prey protein causes activation of the *LEU2* reporter gene.  $\gamma$ -Synuclein interacting clones were able to grow in the absence of leucine as shown in Figure 9A-B, row 1. X-Gal indicates a colorimetric assay in which interaction between  $\gamma$ -synuclein and prey protein allows activation of the *LACZ* reporter gene. Under induced conditions, protein-protein interaction between  $\gamma$ -synuclein and *Drosophila* open reading frame prey protein results in blue coloring of yeast patches after reaction with  $\beta$ -galactosidase (Figure 9A-B, row 2).

#### G. Establishment of Gamma-Synuclein Transgenic Mouse Model in Ovarian Tumorigenesis.

To help unravel the role of  $\gamma$ -synuclein in tumor initiation and progression and establish models to study the effects of inhibitor peptides, we recently establish a transgenic mouse model that over-expressed  $\gamma$ -synuclein in ovarian epithelial cells. To obtain ovarian-specific transgenic mice, we have derived transgenic mice that over-express  $\gamma$ -synuclein under MISIR promoter, an ovarian-tissue specific promoters. Three mice were genotyped as positive, one of them gave birth to 6 mice, and the other two is still in the breeding. We are breeding the founders to get more offspring and the genotype and gene expression will be examined. Although not part of this application, we believe that these mice will provide another model to assess the potential therapeutic value of inhibitor peptides.

## **C- KEY RESEARCH ACCOMPLISHMENTS:**

### **C.1. "Developing Inhibitors of Ovarian Cancer Progression by Targeted Disruption of the $\gamma$ -Synuclein Activated Migratory and Survival Signaling Pathways".**

- 1.a. Overexpression of  $\gamma$ -synuclein leads to constitutive activation of ERK and Rho/Rac/Cdc42 and down-regulation of JNK activation in response to stress signals or chemotherapy drugs (previous).
- 1.b. Overexpression of  $\gamma$ -synuclein induces stress fiber formation and enhances cell migration. Both the basal level and the enhanced cell migration require the activities of both the ERK and Rho/Rac/Cdc42 kinases (previous).
- 1.c. Overexpression of  $\gamma$ -synuclein may render cancer cells resistant to Taxol and vinblastine by modulating ERK cell survival pathway and JNK-mitochondria-Caspase 9/3 apoptotic pathway (previous).
- 1.d. Identified 53 peptide aptamer clones coding for proteins that demonstrated reproducible interactions with  $\gamma$ -synuclein as assayed.
- 1.e. Determine that the vast majority of peptide aptamers contain a premature stop codon, suggesting that the N-terminal portion of thioredoxin may interact with  $\gamma$ -synuclein.
- 1.f. Demonstrated a potential interaction between  $\gamma$ -synuclein and p84N5 (TRESK), a protein that is over-expressed in both breast and ovarian tumors and is essential for the transport of mRNAs from the nuclear to the cytoplasm.
- 1.g. Initiated a second screen to identify additional interactors of  $\gamma$ -synuclein that can be used to assess the activity of the peptide aptamers.



**D-REPORTABLE OUTCOMES (4/2003 to present):**

**D.1.** "Developing Inhibitors of Ovarian Cancer Progression by Targeted Disruption of the  $\gamma$ -synuclein Activated Migratory and Survival Signaling Pathways",

**1.a. Abstracts**

Zhong-Zong Pan and **A.K. Godwin**.  $\gamma$ -Synuclein May Render Cancer Cell Resistance to Paclitaxel by Activating AKT. Proceedings of American Association of Cancer Research, 44:2031, 2003.

Pan, Z.-Z., Vanderhyden, B. and **Godwin, A.K.**  $\gamma$ -Synuclein transgenic mouse model in cancer initiation and progression. Proceedings of American Association of Cancer Research, 45:5108, 2004.

Guo, G., Farber, M.J., Shiekhatter, R. and **Godwin, A.K.** Over-expression of death domain containing protein-p84N5 in human ovarian cancer cell lines is associated with cell proliferation. Proceedings of American Association of Cancer Research, 45:1805, 2004.(Selected for an award as an AACR scholar-in-training).

**1.b. Publications**

Gupta, A., **Godwin, A.K.**, Vanderveer, L., Lu, A.P., and Liu, J. Hypomethylation of the synuclein  $\gamma$  gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res.*, 63:664-673, 2003.

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhatter, R., **Godwin, A.K.** Linking Transcriptional Elongation and mRNA Export to Metastatic Breast Cancers, *Cancer Research*, in press, 2005.

Pan, Z-Z., Bruening, W., Giasson, B. **Godwin, A.K.**, Requirement of RHO and ERK Activation in  $\gamma$ -Synuclein Enhanced Breast and Ovarian Cancer Cell Migration and Invasion, *JBC*, under review, 2005.

**Book chapters and review articles:**

Ozols, R.F., Bookman, M., Connolly, D., Daly, M.B., **Godwin, A.K.** Schilder, R., Xu, X., Hamilton, T.C. Focus on epithelial ovarian cancer. *Cancer Cell*, 5:19-24, 2004.

Pan, Z-Z., **Godwin, A.K.** Oncogenes. Encyclopedia of Molecular Cell Biology and Molecular Medicine. Edited by R.A. Meyers, Second Edition, Volume 9. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp., 435-495, 2005.

**E-CONCLUSIONS:*****E.1. "Developing Inhibitors of Ovarian Cancer Progression by Targeted Disruption of the  $\gamma$ -Synuclein Activated Migratory and Survival Signaling Pathways "***

In our previous studies, we found that  $\gamma$ -synuclein can interact with two major MAPKs, i.e., ERK and JNK1. Over-expression of  $\gamma$ -synuclein may lead to enhanced activity of ERK and down-regulation of JNK activation in response to stress and chemotherapy drugs. Rho/Rac/Cdc42 pathway is also activated in cells over-expressing  $\gamma$ -synuclein. Activation of both the Rho/Rac/Cdc42 and ERK pathways are required for the enhanced cell migration in  $\gamma$ -synuclein over-expressing cells. Over-expression of  $\gamma$ -synuclein may render cancer cells resistant to Taxol and vinblastine by modulating ERK cell survival pathway and JNK-mitochondria-Caspase 9/3 apoptotic pathway. Taken together, these data indicate that  $\gamma$ -synuclein may promote tumorigenesis by enhancing cell motility through modulating Rho/Rac/Cdc42 and ERK pathways, and promoting cell survival and inhibiting apoptosis through modulating ERK cell survival and JNK-mitochondria-caspase9/3 apoptotic pathways. Since  $\gamma$ -synuclein is aberrantly expressed in the majority of late-stage ovarian cancers but is not expressed in normal ovarian epithelial cells,  $\gamma$ -synuclein may represent a very promising therapy target for these diseases. In this aspect we have uncovered a number of peptide aptamer sequences that appear to interact with  $\gamma$ -synuclein. Furthermore, we have identified that  $\gamma$ -synuclein may interact with TREX84, an important protein which is overexpressed in breast and ovarian tumors and is involved in mRNA metabolism. Studies are currently underway to express these various peptides in yeast cells to examine if they lead to disruption of  $\gamma$ -synuclein interactors.



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**APPENDICES**

None